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AQUATIC PLASTIC POLLUTION

Plastics underground: microplastic pollution in South African freshwater caves and associated biota

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Abstract Microplastics (MPs) have been characterised in South African rivers, lakes, and the marine environment, yet we know less about MPs in subterranean environments. In this study, we assessed MP pollution in the sediment, subsurface water, and resident freshwater amphipod, *Sternophysinx* species across six South African subterranean cave systems. We hypothesised that MP pollution will increase with human visitations and activities in and around selected subterranean caves. We found MPs in sediments, subsurface waters, and amphipod species ranging from 4.9 ± 1.2 to 25.0 ± 6.9 particles/kg^{-1}, 2.7 ± 0.7 to 15.0 ± 1.7 particles/L^{-1} and 2.1 ± 0 to 9.8 ± 3.1 particles/dry mass, respectively, with polypropylene being the most abundant polymer according to FTIR analysis. White fibres were dominant in sediments and water samples, whereas blue fibres were dominant in amphipods. Our results supported the hypothesis that MPs densities were correlated with human visitation and activities in and around the caves. The presence of MPs in subterranean caves presents a biodiversity and conservation threat to endemic and understudied cave-dwelling aquatic invertebrates, due to MPs ability to be transferable between trophic levels causing physiological constraints.

Keywords Subterranean environments · Groundwater pollution · *Sternophysinx* species · Trophic transfer · Anthropogenic activities

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Introduction

Microplastic (MP) pollution has gained significant attention in the past decade due to its wide distribution and ecological impacts including potential harm to humans, marine, and freshwater organisms, causing trophic disruptions and biodiversity loss (Ogonowski et al., 2018; Ma et al., 2020; Rochman & Hoellein, 2020; Pinheiro et al., 2021). Global plastic production has expanded rapidly, from 1.5 million tons in the 1950s to 390.7 million tons in 2022 (PlasticsEurope, 2022). According to Boucher & Friot (2017), the largest contribution of MP pollution originates from primary sources such as cosmetics, clothing, and personal care products (hereafter referred to as primary MPs). Considering their durability, persistence, and small size of < 1 μm–5 mm (Frias & Nash, 2019), primary MPs can easily be transported unaidedly into and between aquatic habitats, including groundwater habitats (Singh & Bhagwat, 2022), where they can pose a significant threat to some unique and sensitive aquatic ecosystems.

Groundwater is a vital component of the Earth's hydrological cycle and comprises approximately 30% of the world's readily available freshwater reservoirs (Todd & Mays, 2004). This ecosystem plays a crucial role in sustaining surface freshwater resources and biodiversity (Griebler et al., 2015), maintaining aquafer storage (Foster et al., 2003), and regulating the water cycle (Miguez-Macho et al., 2012). However, these ecosystems face numerous threats due to landscape developments taking place above ground including contamination by industrial, domestic, and agricultural waste and activities and overexploitation, which can lead to poor water quality, thus affecting the availability potable freshwater resources (Singh & Bhagwat, 2022). The presence of MPs in groundwater has been shown to have a negative impact to aquatic organisms including microorganisms, invertebrates, fish, humans, domestic, and wildlife, through bioaccumulation, and overall disrupting the ecosystem functions (Sforzi et al., 2024). It is also worth noting that conservation efforts such as sustainable groundwater management and effective wastewater treatment have been made to mitigate groundwater contamination issues (Wynne et al., 2021; Mammola et al., 2022; Khant and Kim, 2022). The introduction of MP in groundwater highlights the need for comprehensive research and urgent action to address this emerging environmental issue.

It is estimated that 11% of the plastic produced every year ends up in aquatic ecosystems (Borrelle et al., 2020). Therefore, monitoring MP types and pollution in the environment is essential to comprehend their potential sources and fate (Barboza et al., 2018; Henry et al., 2019; Prata et al., 2019). Microplastic has been found in marine (Naidoo et al., 2015) and various surface freshwater waterbodies including rivers (Nel et al., 2018; Jiang et al., 2019; Dalu et al., 2021), lakes, and reservoirs (Mbedzi et al., 2020; Mutshekwa et al., 2023; Nava et al. 2023), with evidence showing direct and indirect MPs ingestion by several aquatic organisms (Redondo-Hasselerharm et al., 2018a, b; Windsor et al., 2019; Gallitelli et al., 2024). However, there is little research on MP pollution in groundwater (Schimdt & Hahn, 2012; Jasechko & Perrone, 2021), despite groundwater's contribution to potable freshwater resources maintenance, aquatic biodiversity, and ecosystem health (Saccò et al., 2019). Microplastics in groundwater are often discussed in terms of their occurrence (see Panno et al., 2019; Singh and Bhagwat, 2022; Viaroli et al., 2022), with little attention given to the potential effects on organisms within these connected ecosystems (Storzi et al., 2024). Although subterranean freshwater environments are progressively perceived for their ecological and socio-economic value (Cantonati et al., 2020), they have not yet been sufficiently documented and examined concerning MP pollution in comparison with their counterparts i.e. surface freshwater bodies and marine ecosystems (Panno et al., 2019). Similar to surface freshwater bodies, subterranean freshwater caves are equally vulnerable to environmental change and degradations; they can be effectively damaged by pollution contamination (Mammola et al., 2019; Sánchez-Fernández et al., 2021), causing an irreparable loss of natural habitats and endemic species (Khatri and Tyagi, 2015). For instance, assessing MPs pollution in freshwater caves is crucial, since they not only known to be important freshwater water reserves (see Moldovan et al., 2020), and/or hosting unique, rare, and endemic biodiversity, but they are also well known for their paleontological and geological heritage importance, popular particularly for their peculiar speleothems (Culver and Pipan, 2019.

Microplastic pollution sources in caves can include waste and storm water runoff, atmospheric deposition, and physical littering (Panno et al., 2019). The pollution and distribution of MPs in both aquatic and terrestrial environments can have far-reaching consequences, where they can be transported vertically into the subsoil, travelling over long distances throughout the rock fractures, and accumulating in the groundwater system (Chia et al., 2021), thus further contaminating groundwater reserves and ultimately finding their way to subterranean freshwaters. As of late, the interest in subterranean environments has grown remarkably, highlighting the importance of biodiversity conservation and sustainable management of such important and unique environments (Chiarini et al., 2022). However, subterranean freshwater environments in Southern Africa are neglected, yet they are home to a diverse range of species, some of which are endemic and highly adapted to these unique, isolated ecosystems. Despite their ecological significance, these environments remain underexplored and are often overlooked in conservation efforts across the region.

For an example, South Africa is home to several subterranean freshwater limestone caves, scattered across the country, of which some are of great paleontological and paleoarchaeological importance (Beaumont & Vogel 2006; Herries et al., 2020). Biologically, these caves support important populations of bats, including the endangered Natal long-fingered bat *Miniopterus natalensis*, and countless numbers of terrestrial animals (Irish & Marais, 2002; Jacobs et al., 2017; Ferreira et al., 2020). In terms of aquatic animals, amphipod communities are known endemics dominating South African cave systems (Griffiths & Stewart, 2001). These caves vary considerably in their human usage and access, some are generally open to the public, while some are more restricted and on private land. Freshwater caves in South Africa face significant environmental challenges, primarily due to pollution and over-extraction of water (Sharrat et al., 2000; Durand et al., 2010). Human visitation, agricultural runoff, industrial waste, and sewage discharge can potentially contaminate these caves, resulting in deteriorating water quality and impacting the health of aquatic organisms. Activities like mining and urban development are known to directly destroy South African cave habitats or alter the hydrological system that sustains them (Durand, 2008; Du Preez 2014; Pretorius et al., 2021). Climate change adds another layer of risk by altering precipitation patterns and increasing the frequency of extreme weather events, which thus destabilise the cave environment and affect water availability (Faith et al., 2019). Currently, freshwater habitats are among the most threatened ecosystems in South Africa (Kajee et al., 2023) and while caves may fall within some of these ecosystems, they have not yet been directly assessed as important ecosystems, nor incorporated into active management or conservation plans at a national level, thus reinforcing the need to protect such habitats.

Currently, there has been no characterisation of MPs pollution in South African freshwater caves and their biota. Thus, filling this research gap is the rationale for the present study, which aims to investigate and characterise (shape, colour, polymer type, and size) the MP occurrence, density in sediments, subsurface waters, and amphipod species of six South African freshwater caves associated with various land-use activities. We hypothesised that MPs would be widespread through all caves and amphipod fauna and that caves with low land-use and limited human access; thus, few human activities or usage will have the least MPs densities and that caves with high land-use and touristic attraction to have the high MPs densities. Furthermore, we hypothesised that caves with higher land use will have a greater diversity of MPs, with a broader range of shapes, colours, and polymer types, compared to caves with minimal human impact. Our research will provide a baseline for future work related to the transport and fate of MPs in South African subterranean environments that can inform mitigation and management plans, particularly if the environment and associated biota are threatened by MPs.

Materials and methods

Study area

The study was conducted in six subterranean freshwater caves in South Africa across a gradient of land use (i.e. Low, Moderate, High) and human activities (i.e. deep water diving, rock climbing, and spiritual activities) (Fig. 1). Kogelbeen and Ficus caves, located in the Northern Cape and Limpopo, respectively, were associated with low land use and human activities due

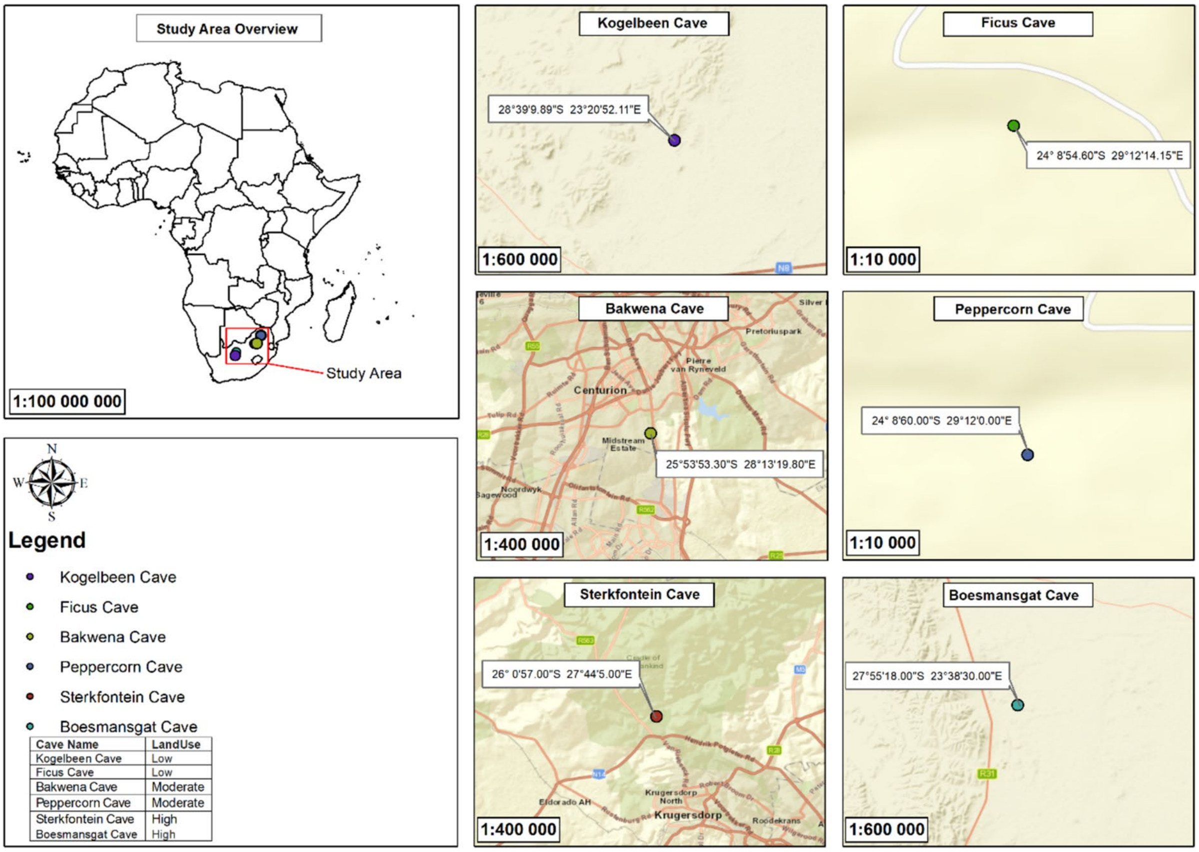

Fig. 1 Study sites locations for the six freshwater caves across three provinces i.e. Northern Cape, Gauteng and Limpopo, South Africa

to their challenging accessibility. In contrast, Peppercorn in Limpopo and Bakwena in Gauteng caves were associated with moderate land use and human activities, where both cave where open to public and ease access, whereas Boesmansgat in Northern Cape and Sterkfontein in Gauteng caves were associated with high land use and human visitation including picnic sites, tourism centres, and exhibition and rock climbing and deep diving.

Kogelbeen Cave, located in the Northern Cape of South Africa (28° 39′ 11.0″ S, 23° 20′ 52.8″ E), is the longest known cave in the region, measuring 788 m in length (from the mouth to its deepest explored point). Situated at an elevation of 1397 m above sea level (Irish & Marais, 2002), it features dolomitic limestone formations and is recognised as South African Natural Heritage Site. The cave entrance leads to a sinkhole and descends to a maximum relative depth of − 8 m. Freshwater, typically found at 57 m, was observed at a lower level during a recent sampling event, likely due to reduced rainfall (Reiss et al., 2019). The cave serves as an important roost for three bat species: *Miniopterus schreibersii, Rhinolophus clivosus*, and *Rhinolophus darlingi* (Herselman and Norton, 1985). It also harbours unique aquatic life, including the stygobiotic amphipod *Sternophysinx basilobata* Griffiths, 1991, known only from Kogelbeen and Boesmansgat caves. Located on private property, the cave is not open to the public and thus has limited human activity.

Peppercorn (24° 09′ 31.0″ S, 29° 10′ 37.0″ E) and Ficus caves (24° 08′ 54.60″ S, 29° 12′ 14.15″ E) are both situated on the northern slopes of the Makapansgat Valley (or Makapan Valley Fossil Hominid Sites of South Africa World Heritage Site, UNESCO), northeast of Mokopane in Limpopo province. The two caves are situated some 150 m apart and potentially share the same ground-water aquifer and are situated top of a dolomite (Patridge, 1966). Both caves have an underground lake with amphipods species including *Sternophysinx alca* Griffiths, 1991, and *Sternophysinx r*obertsi Methuen, 1911. The caves are in a provincial protected area; however, locals use the caves for religious and cultural rituals (Fig. 2).

Bakwena Cave (25° 53′ 53.3″ S, 28° 13′ 19.8″ E) in Gauteng, South Africa, is located within dolomitic rock on the Irene campus of the Agricultural Research Council and is accessible to the public (Jacobs et al., 2017). This sinkhole cave is one of the few dolomitic caves in South Africa with freshwater access and allows entry by rappelling down a cone-shaped sinkhole and descending a 5-m ladder to reach the main cavern (Durang et al., 2012). The cave system includes a large main chamber with three tunnels, one of which leads to a partially submerged chamber, and is surrounded by grasslands that serve as a bat foraging area. It hosts dark-adapted species such as Natal clinging bats (*Miniopterus natalensis*), fungi (*Aspergillus* and *Penicillium*), nematodes, and two amphipod species, *Sternophysinx filaris* and *Sternophysinx calceola* (Mlungu, 2021). The locals use for religious and cultural rituals (Fig. 2).

Boesmansgat Cave, also known as Bushman's Hole (27° 55′ 18″ S, 23° 38′ 30″ E), is a renowned freshwater sinkhole in Northern Cape, South Africa, located on Mount Carmel game farm, about 55 km southeast of Kuruman. This dolomite cave, formed by underground water dissolution, is the world's third-deepest water-filled cave, reaching a depth of 283 m and attracting record-breaking divers (Beaumont & Vogel, 2006; Baglow & Mthembi, 2021). The surface pond, about 100 m wide and often covered by duckweed, is surrounded by semi-arid savannah with species like *Senegalia mellifera* (black thorn) and *Vachellia erioloba* (camel thorn) (Mlungu, 2021). The cave is a home to two co-occurring endemic subterranean amphipod species, *Sternophysinx basilobata* Griffiths, 1991, *Sternophysinx megacheles*

Fig. 2 Aerial view of **a** Boesmansgat submerged freshwater cave and plastic pollution observed in **b** Bakwena cave, **c** Peppercorn cave, and **d** Ficus cave during sample collection

Griffiths and Stewart, 1995 (see Griffiths and Stewart (2001) (Table 1). Visitors descend a steep rock face to reach the water, where the narrow entrance opens into a large chamber extending over 200 m deep. The cave experiences pollution from atmospheric pollutants and tourism activities (Fig. 2).

Sterkfontein Cave is part of the UNESCO Fossil Hominid Sites in the Cradle of Humankind, Gauteng, South Africa (26° 00′ 57″ S, 27° 44′ 05″ E), and lies 50 km northwest of Johannesburg. This limestone cave system, renowned for its ancient fossil deposits within dolomitic rock (Brink & Partridge, 1965), includes a groundwater-fed subterranean lake with a water level at approximately 1440 m above sea level (Hobbs & Meillon, 2017). The main cavern is accessible by a 15-m stairway (Tasaki, 2006; Stratford, 2017) and is surrounded by smaller caves and sinkholes, including Lincoln Cave (Reynolds et al., 2003, 2007). The cave hosts diverse species, including the wasp *Belonogaster petiolate* (Keeping, 1990), and is the type locality for the freshwater amphipod *Sternophysinx filaris* Holsinger & Straškraba, 1973 (Mlungu, 2021). Privately owned and managed, Sterkfontein offers guided tours and remains a valuable site for paleontological research, though it was temporarily closed for eight months due to unstable conditions and repairs following adverse weather.

Table 1 Summary of main features of examined freshwater caves in South Africa: Boesmansgat cave, Kogelbeen cave, Sterkfontein cave, Bakwena cave and two Makapan's caves (Peppercorn and Ficus caves)

Cave name	Distance from the surface to the freshwater	Distance to the closest city	Nature of the cave	Access (public or private)	Main human activity	Endemic amphipods species
1. Boesmansgat cave	300 m	26.56 km	Aboveground (Sinkhole)	Private, with restricted access	Diving, picnic, rock climbing	Sternophysinx basilobata Griffiths, 1991 *Sternophysinx megacheles* Griffiths and Stewart, 1995
2. Kogelbeen Cave	300 m to the isolated pools and 450 m to the big water pool	22.75 km	Underground	Private, access very restricted	no human activities, cave is highly secluded	*Sternophysinx basilobata* Griffiths, 1991
3. Sterkfontein cave		6.83 km	Underground	Public, relatively easy access	Tourist & paleo-research	*Sternophysinx filaris* Holsinger & Straškraba, 1973
4. Bakwena cave		249.75 m	Underground	Public now, used to be private, access is now easy	Religious & cultural rituals	*Sternophysinx filaris* Holsinger & Straškraba, 1973 *Sternophysinx calceola* Holsinger, 1992
5. Peppercorns cave		4.63 km	Underground	Public, with restricted access	Religious and cultural rituals	*Sternophysinx alca* Griffiths, 1981 and *Sternophysinx robertsi* Methuen, 1911
6. Ficus cave		4.63 km	Underground	Public, with restricted access	Religious, cultural rituals & paleo-research	*Sternophysinx alca* Griffiths, and *Sternophysinx robertsi* Methuen, 1911

Sampling collection and extraction

A total of 18 (1.5–2.0 kg) sediment samples (i.e. 6 sites × 3 replicates = 18 sediments samples) were collected at three randomly selected stations near the water line at a depth of 5.0 ± 0.5 cm using a steel hand shove. Sediment samples were then covered with an aluminium foil, transferred into a new labelled Ziplock bags, and transported to the laboratory for further pre-treatment and analysis. Using a 25L stainless-steel bucket (305 mm diameter), 100 L of subsurface water (equivalent to four 25 L buckets) was randomly collected at a depth ranging from 5 to 20 cm below the water surface and filtered on-site through a stacked four nylon mesh sieves of different mesh sizes of 1000, 500, 250, and 100 µm, respectively. This process was repeated three times per at each cave (6 sites × 3 replicates = 18 water samples) following a similar approach by Qu et al. (2018). Thereafter, all four nylon mesh sieves were immediately covered with aluminium foil to prevent airborne contamination until they reached the laboratory.

To collect resident amphipod specimens, a handheld squared-shaped aquatic net (frame 30 cm × 30 cm, mesh size 500 µm) and Multinet sampler (0.25 m^2 opening and with the standard 200 µm mesh; Hydro-Bios Kiel, Germany) were used. Amphipods from the genera *Sternophysinx* were chosen to assess MP ingestion since they are known endemic aquatic invertebrates species found in subterranean system and they are of conservation concern, thus ideal candidates to compare MP ingestion and impacts (Blarer et al., 2016). At each site ($n = 6$), a total of 9 amphipods individuals (3 individuals per replicate) of similar size were collected randomly; however, this was not the case for the Boesmansgat Cave, where only a total of 5 amphipods were found and placed in a single vial. The number of amphipod samples was due to the low density across the caves. After collection, amphipods were immediately preserved in 80% ethanol solution, following Courtene-Jones et al. (2017), and then transported to the laboratory.

In the laboratory, all sediment samples were weighed and then added to a 25 L stainless-steel bucket, separately, and then filled with NaCl solution (made by dissolving 1400 g NaCl in 0.6 L, density 1.22), following the density separation method which was previously reported by Nuelle et al. (2014) and Quinn et al. (2017). The mixture was stirred vigorously for 6 min, allowing the less dense MP particles to float to the surface (Lusher et al., 2015). After settling, the supernatant was passed, through four stacked nylon mesh sieves with different sizes of 1000, 500, 250, 100 µm, respectively, and the process was repeated six times for each sample, to effectively remove available MPs from the fine sediment following Naidoo (2018). Thereafter, the nylon mesh sieves were oven-dried at 30 °C overnight prior to MP identification under a dissecting microscope. Each sieve was placed under a dissecting microscope at X50 magnifications, and MP particles observed were counted and characterised into type, size, and colours. The water sieves were also oven-dried at 30 °C overnight and thereafter inspected for MP following similar process as described above.

The collected amphipod specimens, 3 individuals per replicates were placed in three different petri dishes, weighed, and then oven-dried at 60 °C for 24 h where the total mass was noted. Oven-dried amphipods samples (three replicates of 3 dried individuals) were further placed into a single 350-mL glass vial containing 1 mL of nitric acid, including a combined 5 individuals from Boesmansgat cave and digested overnight at room temperature (Silva et al., 2022). The solution was then diluted with distilled water and filtered through Whatman membrane filters (2 µm mesh pore; 47 mm diameter); thereafter, filters were allowed to dry overnight at room temperature. Microplastic particles were identified using a microscope as described for sediments and subsurface water samples above. Microplastic particles found were reported in dry mass.

For all samples, MP particles were deemed to be MP if they possessed unnatural colouration (e.g. bright colouration, and multicoloured) and/or unnatural shape (e.g. sharp edges, perfectly spherical see Picó & Barceló, 2019). Microplastics found in samples were characterised according to types and colours following Lusher et al. (2013) and were further photographed under a Nikon ECLIPSE Ti Series inverted microscope, fitted with a DS–US camera powered by NIS–Elements BR software. However, MP retrieved from amphipods were not categorised by various size but instead by the mesh size of the membrane filter used (2 µm).

Laboratory quality control

To prevent contamination during sample preparation in the laboratory, precautionary measures were put in place during MP extraction and identification process (Bhat et al., 2024a, b). All surfaces and equipment used were rinsed with milli-Q distilled water and then dried overnight before use (Gallitelli et al., 2020). Cotton laboratory coats and polymer-free gloves were used during the laboratory to keep the process sterile; this also included avoiding the use of air-conditioners to minimise the potential risk of air-borne MP particle contamination. Filtration and identification also occurred under a laminar flow hood cabinet to restrict airborne contamination. To determine whether contamination was a confounding factor under laboratory conditions, blanks of glass microfiber filters were situated as follows: (1) open inside the laminar; (2) open in the oven, and (3) open in the laboratory next to the microscope (following Davison and Asch, 2011). Laboratory blanks were used as a "negative control" to cross-check the contamination of plastic materials. Any MP particles found in the blanks were subtracted from our results following Taurozzi et al. (2024). Extraction efficiencies were assessed by filtering known quantities of particles to verify the recovery rates of MPs, following the recommendations of Dimante-Deimantovica et al. (2022). The recovery process demonstrated an efficiency rate exceeding 95%.

Fourier transform infrared spectroscopy analyses

Fourier transform infrared (FTIR) analyses were used to confirm visual inspections of MPs (Mintenig et al., 2019). Prior to FTIR analysis, selected MP particles were treated with potassium hydroxide (KOH) for 1 h to digest any organic matter while leaving MP particles intact (Prata et al., 2019). Once the organic matter was successfully removed, the MP particles were dried thoroughly before FTIR to prevent interference from moisture. Therefore, a random subsample of MP particles, following (Martin et al., 2017), with sizes between 100 and 1000 µm were selected for polymer identification using a vibrational Platinum-ATR Fourier transform infrared spectroscopy (FTIR-ATR) (Bruker Alpha model, Germany). The analysis was conducted with a spectral range of 650–4000 cm^{-1}, at a resolution of 8 cm^{-1}, and 16 scans per analysis. Prior to each sample analysis, background scans were performed, and the ATR crystal was cleaned with 70% propanol. All obtained spectra were compared and verified against the following databases: Hummel Polymer Sample Library, HR Polymer Additives and Plasticizers, HR Hummel Polymers and Additives, and Synthetic Fibres by Microscope.

Statistical analyses

A parametric analysis of variance (ANOVA) was used to assess differences in MP densities (sediments, subsurface waters) between the six freshwater caves after confirmation for homogeneous variances (Levene's test, $p < 0.05$) and normality (Shapiro–Wilk test, $p > 0.05$). Additionally, a post hoc Tukey multiple comparison test was performed to identify which caves showed significant differences. All statistical analyses were performed using R Development Core Team (2018). Pearson rank correlation analysis was used to investigate the relationship between sediment and water MP densities and amphipods *Sternophysinx sp.* MP densities using SPSS version 16.0.

Results

Microplastics in sediments, subsurface water, and amphipods

All control samples contained no MPs, whereas MPs were found in all sediment and water samples, with an overall average density of 13.5 ± 2.0 particles/kg^{-1} and 11.01 ± 1.30 particles/L^{-1}, respectively. Microplastics ranged between 4.9 ± 1.2 and 25.0 ± 6.9 particles/kg^{-1} (Fig. 3A) and 2.7 ± 0.7 and 15.0 ± 1.7 particles/L^{-1} (Fig. 3B) in sediments and water, respectively. Boesmansgat cave had the highest MP particles for both sediments (25.0 ± 6.9 particles/kg^{-1}) and water (15.0 ± 1.7 particles/L^{-1}), whereas Kogelbeen cave had the least MP particles for both sediments 4.9 ± 1.2 particles/kg^{-1} and water 2.7 ± 0.7 particles/L^{-1}. Overall, MPs from the sediment samples were significantly different between sites ($F = 3.987$; $df = 5$; $p = 0.0023$), where only Boesmansgat cave was significantly different to Kogelbeen and Bakwena caves (Fig. 3A). Significant differences were also observed for water samples between caves ($F = 3.266$; $df = 5$; $p = 0.0432$) (see Fig. 3B).

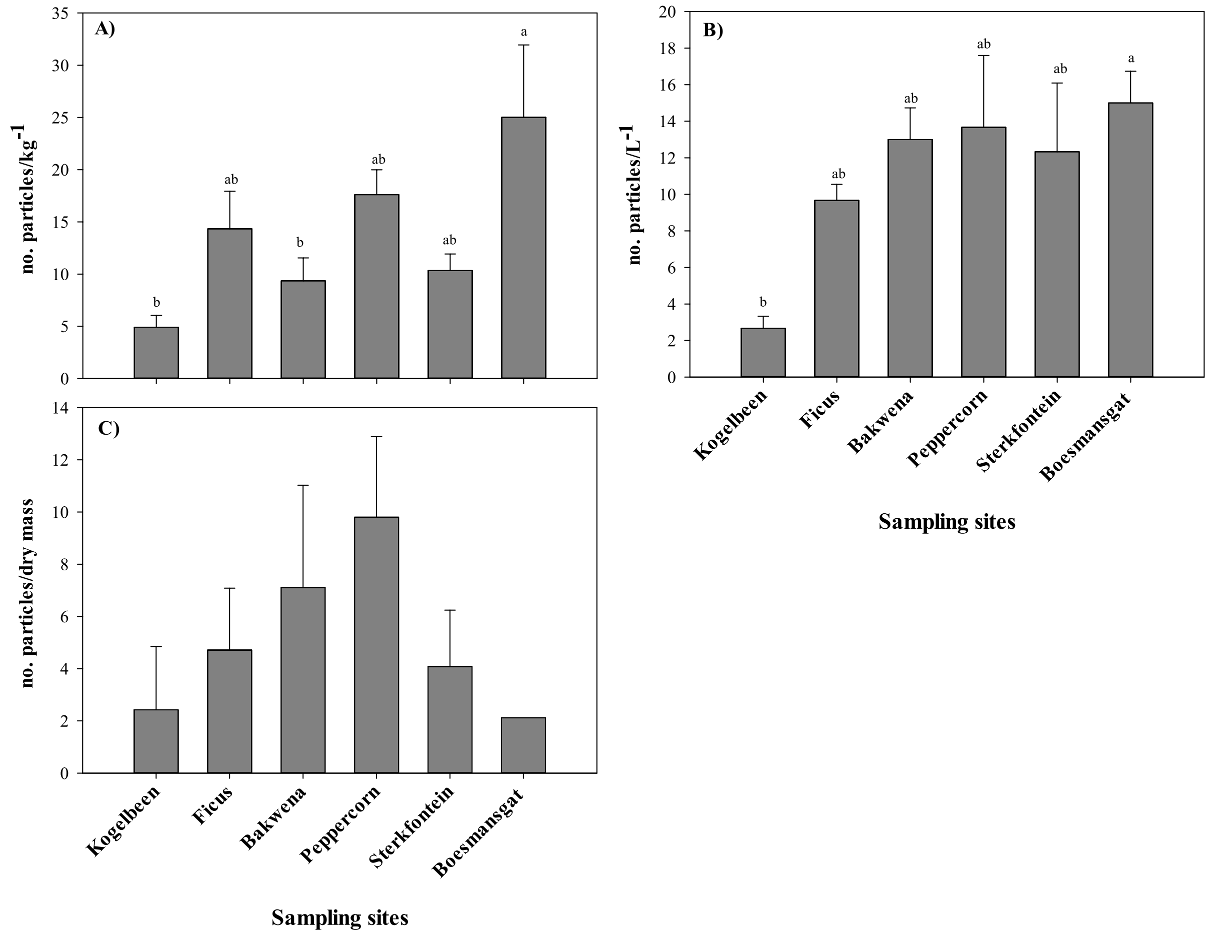

Fig. 3 Microplastic particles (Mean ± SD) in **a** sediments, **b** water, and **c** amphipods across six freshwater caves in South Africa

Of 50 *Sternophysinx sp.* specimen collected across six caves, 38 (76%) amphipods contained MP particles. Microplastic particles in amphipod samples had an overall average density of 5.41 ± 1.2 and ranged between 2.1 ± 0 and 9.8 ± 3.1 particles/dry mass (Fig. 3C). The highest MP particles of 9.8 ± 3.1 particles/dry mass were found in Peppercorn cave, and the lowest MP particles of 2.1 ± 0 particles/particles/dry mass were recorded in Boesmansgat cave (Fig. 3C).

Based on Pearson correlations, there was no significant relationship ($p > 0.05$) observed for MP densities found within amphipods versus sediment and water MP densities (Fig. 4). Positive, non-significant correlations were observed for amphipods MP densities (Pearson $r = 0.406$; $p = 0.095$) and water MP densities (Pearson $r = 0.406$; $p = 0.095$) (Fig. 4B), whereas negative non-significant correlations were observed for amphipods MP densities ($r = -0.17$, $p = 0.004$) and sediment MP densities ($r = -0.096$, $p = 0.705$) (Fig. 4A).

Morphological characteristics

Microplastic particle's shape, colour, and size

Fibres, filaments, and fragments were the most common MP particles found in the sediments, water, and amphipod samples (Fig. 5). A variety of MP shapes were found in sediment samples across six caves, with fibre and filament accounting for 54.2% of the total abundance of MPs, followed by fragment, pellet, foam, and film accounting for 30.8%, 8.6%, 3.6%, and 2.7%, respectively (Fig. 5A). In water samples,

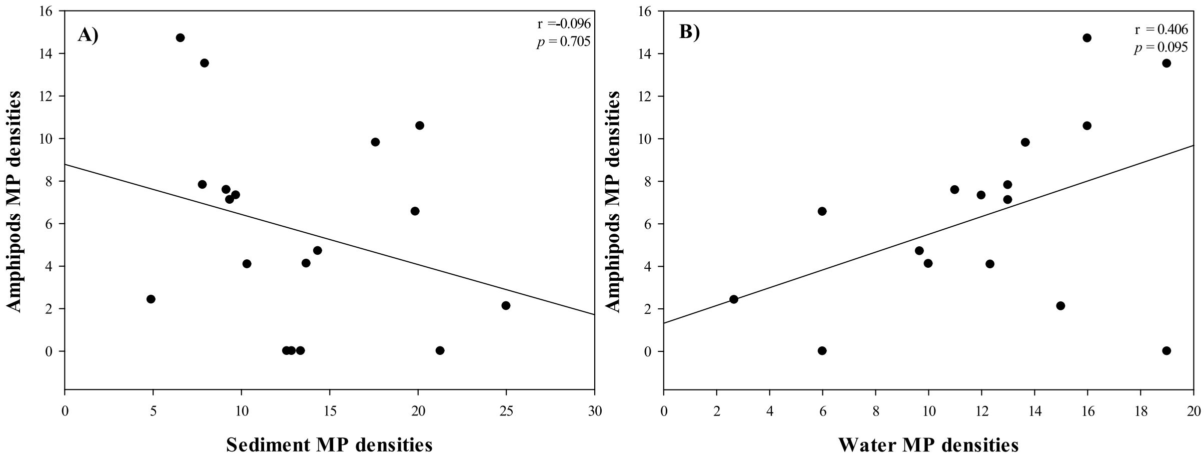

Fig. 4 Relationship between MP densities in amphipods versus **a** sediment, and **b** water

fibre and filament were the most dominant MP shape, accounting for 67.1% of the total percentage composition of MP shapes, followed by fragment, pellet, foam, and film, which accounted for 19.1%, 6.4%, 4.0%, and 3.5%, respectively (Fig. 5B). In amphipod samples, three MP shapes were found i.e. fibre and filament accounting for 79.5%, followed by fragment with 20.5% of the total percentage composition of MP (Fig. 5C).

White MPs dominated both sediment and water samples, followed by black and transparent, while in amphipod samples, blue was the most identified, followed by transparent, black, and brown (Fig. 5). In sediment samples, white particles accounted for 33.1% of the total densities across caves, followed by transparent and black accounting for 23.4% and 20.0%, respectively (Fig. 5A). Water samples also demonstrated a variety of colours across caves, with white accounting for 27.3% of the total MP densities, followed by black (26.1%) and transparent (23.9%) particles (Fig. 5B). Microplastics found in amphipods were predominantly blue and white, accounting for 48.7% and 20.5%, respectively, of the total MP densities across caves (Fig. 5C). The FTIR-ATR results on selected MP particles showed that the majority of particles were represented by six polymer types i.e. polypropylene, polystyrene, polyvinyl chloride, polyethylene, polydimethylsiloxane, where polypropylene (38%) was the most dominant and the least been polydimethylsiloxane (4%).

Most MP particles in sediments were found to be around 100 µm in size (range 32.6–45.2%), followed by 250 µm (range, 13.0–29.2%), 500 µm (range, 19.9–26.2%), and 1000 µm (range, 11.3–17.6%) (Fig. 6A). Microplastic particles from water samples followed a similar trend with different percentage ranges, that is 1000 µm (range, 12.1–27.0%), followed by 500 µm (range, 7.9–27.6%), 250 µm (range, 6.7–32.4%), and 100 µm (range, 18.9–51.1%) (Fig. 6B).

Discussion

Quantifying and characterising MP pollution in subterranean freshwater caves is crucial for establishing their impact on important below-ground habitats and water resources. South African subterranean freshwater caves in particular are not well investigated, yet they are important systems that support unique biodiversity and host important organisms such as cave-adapted crustaceans. Microplastic abundance in South African caves was found to vary across the caves and thus partially supported our hypothesis. Microplastic particles were present in sediments, subsurface waters, and amphipods in all caves, highlighting the ubiquitous nature of MP pollution and its potential to be found even in the most remote and pristine environments. Our findings indicate that these caves are experiencing significant MP pollution, and those with low to moderate land-use and human activity may be at risk of increased MP pollution in the near future. Additionally, a variety of MP shapes, colours, polymer types, and sizes were observed,

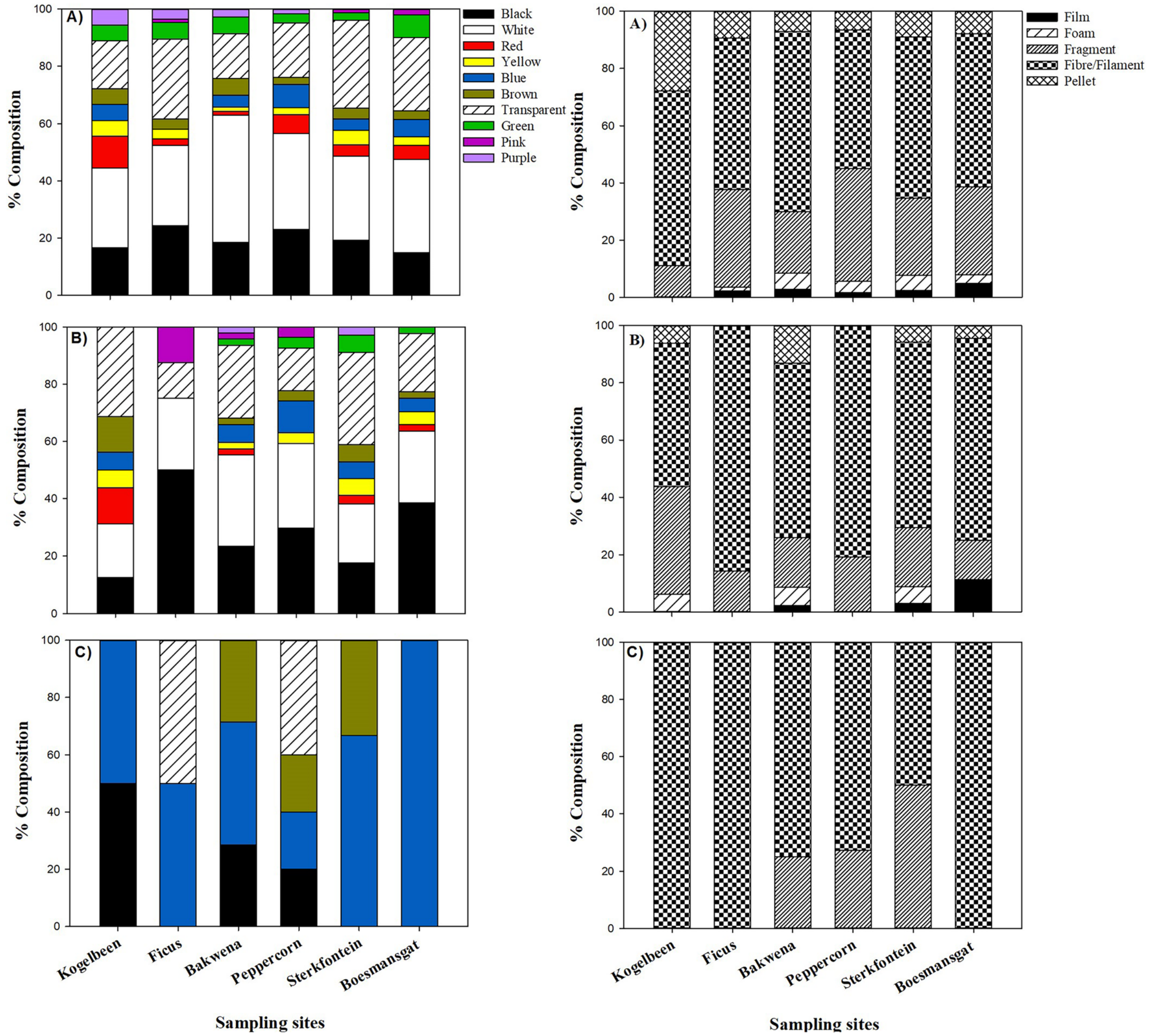

Fig. 5 The proportion of microplastic colours and types found in **a** sediment, **b** water, and **c** amphipods across six freshwater caves in South Africa

suggesting that these particles likely originated from multiple sources. There was no relationship between MP particles in amphipods versus sediment and water. As such, we speculate that amphipods may be exposed to MPs through different mechanisms, possibly by selectively ingesting contaminated food or through direct uptake from the water column, rather than directly from sediments or water.

Only a few studies have examined MPs in sediments and water from subterranean caves globally (Table S1). In the current study, MP particles found in sediments and water samples were higher in Boesmansgat cave, while in the amphipods, MP particles were higher in Peppercorn cave. The increased MP particles in the Boesmansgat cave might be attributed to the fact that the cave is exposed to high land-use activities (e.g. picnic site, deep diving, and rock climbing) and MP atmospheric deposition, and possible flooding/runoff events since the cave is a sinkhole exposed to the landscape (Wright et al., 2020). High MP particles were also found in Bakwena cave, Peppercorns cave, and Ficus cave, which may be attributed to the use of these caves for religious and cultural activities by

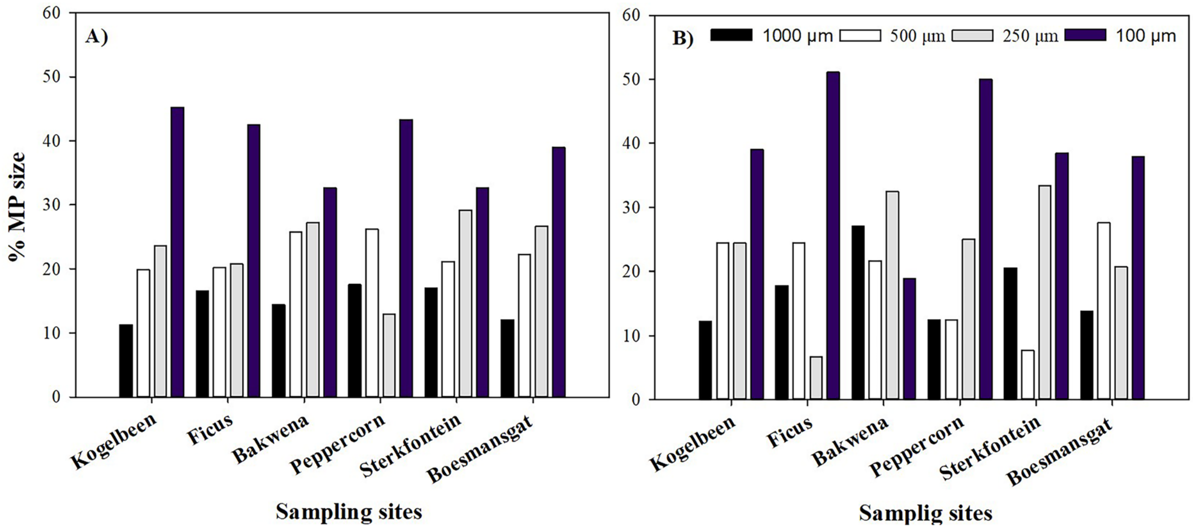

Fig. 6 The percentage of microplastic sizes found in cave **a** sediment and **b** water samples across six caves in South Africa

nearby communities. Sterkfontein cave, which is a tourism exhibition centre and associated with high human visitation in and around, had high MP abundance when compared to Kogelbeen cave which was expected and in agreement to our hypothesis. Reduced MP particles in Kogelbeen cave based on our observations, might be due to limited access to the cave and restriction to the public. The overall MP found in sediments across the caves was lower than those found in other subterranean caves globally (Table S1). For instance, we found MP densities in sediments ranging from 4.9 ± 1.2 to 25.0 ± 6.9 particles/kg^{-1}, whereas few studies have found MP densities higher values than in the current study, for example, Balestra & Bellopede (2022) found between 2500 and 8700 items/kg; Hasenmueller et al. (2023) 842.7 ± 166.4 particles/kg^{-1}; and Balestra & Bellopede (2023) 1033.3–1060 particles/kg^{-1}and 666.7–1103.3 particles/kg^{-1} (see Table S1). The differences in MP densities could be related to limited human access to some caves and reduced surface contamination when compared to tourism caves studied by Balestra and Bellopede et al. (2022, 2023) which are heavily visited by humans. However, MP densities found in water samples (range, 2.7 ± 0.7–15.0 ± 1.7 particles/L^{-1}) corresponded to those found by Valentić et al. (2022) (1.00 ± 1.48 items/m^3 and 9.55 ± 16.64 items/m^3) and Balestra et al. (2023) (12–54 particles/L^{-1}) (see Table S1). This could be related to similar sampling methodologies or the levels of human activity with other studies, suggesting a potential consistency in MP sources and transport mechanisms in subterranean aquatic environments.

The detection of MP particles in sampled amphipods across all caves highlights a widespread contamination issue in subterranean freshwater caves and the potential threat to underground life and potentially entering the food chain. Due to the lack of research on MP ingestion by amphipods in freshwater caves, comparing our results with different studies can be difficult. Amphipods, a key player in cave ecosystems, can ingest these MPs as seen in the current study, possibly leading to physical blockage, reduced food intake, and exposure to toxins. This disruption can cascade through the food web, affecting other species of bats and amphibians that rely on amphipods as a food source, and ultimately threatening the delicate balance and biodiversity of cave ecosystems (Mateos-Cárdenas et al., 2020). Microplastics found in amphipods in the current study did not vary among the caves. The uniform distribution suggests that MPs are pervasive in the broader environment, infiltrating their remoteness or level of human activity. Consequently, it highlights the extensive reach of MP contamination and the necessity for global efforts to address the sources and impact of MPs, even in seemingly pristine and untouched habitats.

Microplastics in sediments, water, and amphipod samples were also observed in different shapes, colours, and sizes. Fibres and filaments were the most dominant types in sediment, water, and amphipods. Our findings corresponded with other studies that found fibres and filament as the most abundant MP types in freshwater caves. For instance, Balestra et al. (2023) found fibres as the most abundant type in the waters of Bossea cave, and Balestra & Bellopede (2023) further observed fibres and filament as the most dominant type in Toirano caves, in Italy. High ingestion of fibres by amphipods corresponds with findings by Jamieson et al. (2019) who found high levels of fibres particles in amphipods. Redondo-Hasselerharm et al. (2018a, b) also reported majority of fibres in aquatic organisms. With regard to colours, white was abundant in sediment and water, and blue particles was predominant in amphipods. Various MP colours often provide visual clues about their origins or sources (Lusher et al., 2020). Different studies have found high white MP particles in sediments (Díaz-Jaramillo et al., 2021; Singh et al., 2021) and waters (Han et al., 2020). Additionally, blue MPs have also been found to be among the most preferred colour by organisms (Rios et al., 2022). The high blue particles in amphipods might be due to blue particles resembling food sources and the ability of blue light to penetrate deeper into water, making them more visible to amphipods, potentially increasing their likelihood of ingestion (Zhang et al., 2021). The MP size variations found were similar to those described for cave sediments (Balestra & Bellopede, 2022). The variation of MP particles offers some insight into their movement in caves. The MP sizes found in sediments were similar to those found in water samples. This might be a result of surface water migrating to the underground environments via sinkholes and rapidly entering the cervices and fractures, thus reaching the underground waters and settling into the sediments. During their movement, larger MP would be impeded, while smaller MP particles would reach the bottom of the cave. Microplastics found in sediments, water, and amphipods included polypropylene and polystyrene. Based on our observation during sampling across the caves, the polymer types found could be due to improper waste disposal, tourism, and the use of plastic packaging in nearby industries and settlements associated with each cave (Gutiérrez-Rial et al., 2024). These polymer types have also been identified in other freshwater caves (see Novruzzade, 2022; Balestra & Bellopede, 2023) and are possible sources from packaging industries, personal care products, and urban sewage. Furthermore, the persistence of these polymers in cave environments highlights limited degradation rates and potential accumulation over time, which may be exacerbated by inadequate waste management practices and occasional flooding events that transport plastic debris from surface areas into subterranean systems.

Microplastic assessment in freshwater caves is essential due to their role as unique and sensitive ecosystems. As indicated by Hasenmueller et al. (2023), freshwater caves, often considered pristine environments, are increasingly threatened by MP pollution. Human usage, atmospheric depositions, and flooding events in the sinkhole caves were observed to be the lead source of MP pollution in the current study. Our findings also suggest that freshwater caves that are not protected and well-managed are potentially at high risk of MP pollution and accumulation. This was evident with Bakwena, Peppercorns, and Ficus caves, which are accessible anytime and are mostly used by nearby communities for religious and cultural rituals. We project that MPs in these caves will be high in the near future due to the persistent degradation of plastic particles initially introduced by humans. Over time, these particles, often derived from synthetic textiles and other human-made materials, will continue to break down into smaller fragments, thus increasing the MP pollution within these secluded caves. Microplastics were found in less utilised and inaccessible caves, such as Kogelbeen cave, indicating the ubiquitous nature of MP pollution, and raising concerns about the extent of widespread distribution and persistence of plastic debris in subterranean ecosystems. With Kogelbeen cave being exposed to flooding events, flooding will significantly continue to contribute to MP pollution by transporting surface contaminates from surrounding areas, carrying them through the surface runoff and depositing them in the cave sediment and water, and eventually getting ingested by aquatic biota.

Further studies assessing MP ingestion in freshwater caves are essential to elucidate MP impacts in subterranean aquatic organisms. Microplastic ingestion in rivers, lakes, and wetlands is also essential to understanding the broader environmental and ecological impacts of MP pollution, as these freshwater systems are key habitats for diverse species. For instance, MP ingestion by amphipods could have implications for higher tropic levels within the ecosystem, thus highlighting the potential ecological consequences of plastic pollution in freshwater caves. Freshwater caves are often neglected when implementing conservation measures. These freshwater caves provide water to various inland environments, sustaining aquatic life, as well as contributing to the overall water cycle, and providing resources for surrounding habitats. As such, assessing the potential threat of MPs in freshwater caves and encouraging conservation measures, while providing recommendations for future studies is required.

Like other ecological studies, this research faced several limitations. Sampling methods were adapted from the literature due to a lack of standardised protocols, which may limit comparability with studies using different methods and units. Additionally, the mesh size used in this study may not have captured all MP since MP ranges from 1 to < 5-micron particles, potentially underrepresenting the overall MP load. Low amphipod densities also restricted us to collecting only a small number of individuals per cave to avoid impacting local populations. Amphipods were treated with nitric acid to analyse MP ingestion, which can degrade or alter certain polymers (Roch & Brinker, 2017; Gulizia et al., 2022), potentially underrepresenting the diversity and abundance of MPs. Future studies should consider alternative digestion protocols, such as potassium hydroxide (KOH) or enzymatic methods, to better preserve a range of polymer types. Nonetheless, our results provide a solid evidence and insight together with a foundation for understanding the occurrence, distribution, and concentrations of MPs in freshwater caves, providing essential baseline data for future research and method standardisation to assess MP pollution in these ecosystems.

Conclusion

To the best of our knowledge, this is the first study to investigate MPs pollution in sediments, subsurface waters, and amphipods across six South African freshwater caves. Microplastics were found in sediments, subsurface waters, and amphipods and vary based on human usage, with caves associated with high human usage having highest MP densities. Given the presence of various MPs shapes, colours, sizes, and polymer types, the sources of the MP particles found in the caves strongly suggest they originated from various pollutants; however, surface atmospheric deposition, runoff, tourists, and religious and cultural rituals may also be a source based on our observations. If these caves progressively get damaged to such a degree that their unique species and delicate ecosystems are destroyed, it will result in the disappearance of irreplaceable biodiversity and loss of vital natural habits. Future studies are needed to document MP dynamics, identifying the sources and fate in more freshwater caves. Surface and subterranean environments are closely connected; therefore, greater efforts should be made to establish more comprehensive measures of protection. Here we have also shown that amphipod specimens from the genus *Sternophysinx* sp. accumulate MPs and that there is no relation between sediment and water MP densities and those found within the amphipods. Further studies should investigate the potential pathways of MPs accumulation in amphipods, including the role of trophic transfer, ingestion behaviour, and habitat preferences.

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Author contributions TM helped in conceptualisation, formal analysis, investigation, methodology, software, writing—original draft, writing—review and editing. SNM contributed to conceptualisation, investigation, methodology, validation, visualisation, writing—original draft, writing—review and editing. TN was involved in formal analysis, software, validation, visualisation, writing—original draft, writing—review and editing. ZM helped in methodology, validation, visualisation, writing—original draft, writing—review and editing. MCM contributed to conceptualisation, formal analysis, investigation, methodology, funding acquisition, writing—original draft, writing—review and editing.

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Data availability Data generated or analysed during this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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